PROCESS FOR THE PREPARATION OF L-AMINO ACIDS BY USING CORYNEFORM BACTERIA

[0001] BACKGROUND OF THE INVENTION

The invention provides a process for the preparation of L-amino acids, in particular L-lysine, by using coryneform bacteria in which the pfkA gene coding for 6-phosphofructokinase and/or the pfkB gene coding for 1-phosphofructokinase are/is attenuated. All references cited herein are expressly incorporated by reference. Incorporation by reference is also designated by the term "*I.B.R.*" following any citation.

[0002] L-amino acids, in particular L-lysine, find application in human medicine and in the pharmaceutical industry, in the food industry and, quite especially, in animal nutrition.

[0003] It is known that amino acids are prepared by fermentation of strains of coryneform bacteria, in particular Corynebacterium glutamicum. On account of its great importance, work on improving the production process is constantly in progress. Improvements to the process may concern measures pertaining to fermentation technology, such as, for example, stirring and provision with oxygen, or the composition of the nutrient media, such as, for example, the sugar concentration during fermentation, or the reprocessing into product-form by, for example, ion-exchange chromatography, or the intrinsic output properties of the micro-organism itself.

[0004] With a view to improving the output properties of these micro-organisms, methods of mutagenesis, selection and mutant selection are adopted. In this way, strains are obtained that are resistant to antimetabolites such as, for example, the lysine analogue S-(2-aminoethyl)cysteine or

that are auxotrophic in respect of metabolites of regulatory significance and that produce L-amino acids.

[0005] Methods pertaining to recombinant DNA technology have also been employed for a number of years for the improvement of strains of Corynebacterium glutamicum producing L-amino acid, by individual amino-acid-biosynthesis genes being amplified and by the effect on the production of L-amino acid being investigated.

[0006] The invention provides improved processes for the fermentative preparation of L-amino acids, in particular L-lysine, by using coryneform bacteria.

[0007] BRIEF SUMMARY OF THE INVENTION

When mention is made in the following of L-amino acids or amino acids, these expressions are intended to mean one or more amino acids, including their salts, selected from the group comprising L-asparagine, L-threonine, L-serine, L-glutamate, L-glycine, L-alanine, L-cysteine, L-valine, L-methionine, L-isoleucine, L-leucine, L-tyrosine, L-phenylalanine, L-histidine, L-lysine, L-tryptophan and L-arginine. L-lysine is particularly preferred.

[0008] When mention is made in the following of L-lysine or lysine, these expressions are intended to mean not only the bases but also the salts such as, for example, lysine monohydrochloride or lysine sulfate.

[0009] The invention provides a process for the fermentative preparation of L-amino acids by using coryneform bacteria in which at least the nucleotide sequence coding for 6-phosphofructokinase and/or the nucleotide sequence coding for 1-phosphofructokinase are/is attenuated, in particular switched off or expressed at a low level.

[0010] This invention further provides a process for the fermentative preparation of L-amino acids in which the following steps are implemented:

a) fermentation of the coryneform bacteria producing the L-amino acid, in which at least the nucleotide sequence coding for 6-phosphofructokinase and/or the nucleotide sequence coding for 1-phosphofructokinase are/is attenuated, in particular switched off or expressed at a low level;

b) enrichment of the L-amino acids in the medium or in the cells of the bacteria; and

c) isolation of the desired L-amino acids, whereby constituents of the fermentation broth and/or of the biomass optionally remain in the end product in proportions or in their total quantities.

[0011] BRIEF DESCRIPTION OF THE FIGURES

Figure 1: map of the plasmid pXK99Emob,

Figure 2: map of the plasmid pXK99EmobpfkB.

[0012] The abbreviations and designations that are used have the following significance.

Kan:	kanamycin-resistance gene aph(3')-IIa from Escherichia coli
HindIII	cleavage site of the restriction enzyme HindIII
NcoI	cleavage site of the restriction enzyme NcoI
XbaI	cleavage site of the restriction enzyme XbaI
RP4-mob	RP4 mobilization site

Ptrc	trc promoter
T1	termination region T1
T2	termination region T2
LacIq	lacIq repressor of the lac operon of Escherichia coli
OriV	replication origin ColE1 from E. coli
PfkB	cloned region of the pfkB gene

[0013] DETAILED DESCRIPTION OF THE INVENTION

The strains that are employed preferably already produce L-amino acids, in particular L-lysine, before the attenuation of the pfkA gene coding for 6-phosphofructokinase and/or of the pfkB gene coding for 1-phosphofructokinase.

[0014] Preferred embodiments are to be found in the Claims.

[0015] The term 'attenuation' in this context describes the diminution or switching-off of the intracellular activity of one or more enzymes (proteins) in a micro-organism that are coded by the corresponding DNA, by use being made, for example, of a weak promoter or by use being made of a gene or allele that codes for a corresponding enzyme with a low activity or that inactivates the corresponding gene or enzyme (protein) and by these measures optionally being combined.

[0016] By virtue of the measures of attenuation, the activity or concentration of the corresponding protein is lowered in general to 0 to 75%, 0 to 50%, 0 to 25%, 0 to 10% or 0 to 5% of the activity or concentration of the wild-type protein or of the activity or concentration of the protein in the initial micro-organism.

[0017] The micro-organisms that are the subject-matter of the present invention are able to produce amino acids from glucose, saccharose, lactose, fructose, maltose, molasses, starch, cellulose or from glycerin and ethanol. It may be a question of representatives of coryneform bacteria, in particular of the genus Corynebacterium. In the case of the genus Corynebacterium, in particular the species Corynebacterium glutamicum should be mentioned, which is known amongst experts for its ability to produce L-amino acids.

[0018] Suitable strains of the genus Corynebacterium, in particular of the species Corynebacterium glutamicum, are especially the known wild-type strains

Corynebacterium glutamicum ATCC13032

Corynebacterium acetoglutamicum ATCC15806

Corynebacterium acetoacidophilum ATCC13870

Corynebacterium melassecola ATCC17965

Corynebacterium thermoaminogenes FERM BP-1539

Brevibacterium flavum ATCC14067

Brevibacterium lactofermentum ATCC13869 and

Brevibacterium divaricatum ATCC14020

and mutants and strains prepared therefrom that produce L-amino acids, such as, for example, the L-lysine-producing strains

Corynebacterium glutamicum FERM-P 1709

Brevibacterium flavum FERM-P 1708

Brevibacterium lactofermentum FERM-P 1712

Corynebacterium glutamicum FERM-P 6463

Corynebacterium glutamicum FERM-P 6464 and

Corynebacterium glutamicum DSM 5715.

[0019] It has been found that coryneform bacteria produce L-amino acids in improved manner after attenuation of the gene coding for 6-phosphofructokinase (EC: 2.7.1.11) and/or of the gene coding for 1-phosphofructokinase (EC 2.7.1.56).

[0020] The nucleotide sequence of the gene coding for 6-phosphofructokinase of Corynebacterium glutamicum can be gathered from patent application WO 01/00844 *I.B.R.* under Identification Code RXA00206 as SEQ ID No. 53.

[0021] The nucleotide sequence of the gene coding for 1-phosphofructokinase of Corynebacterium glutamicum can be gathered from patent application WO 01/00844 *I.B.R.* under Identification Code RXA01882 as SEQ ID No. 57.

[0022] The nucleotide sequences are also deposited in the gene bank under Accession Numbers AX064927 and AX064931, respectively.

[0023] The claimed nucleotide sequences of the genes coding for 1-phosphofructokinase and for 6-phosphofructokinase, represented in SEQ ID No. 3 and SEQ ID No. 1, respectively, are elongated in comparison with the sequences known from the state of the art by, in each instance, preferably up to 700 base-pairs in front of the start codon and behind the stop codon of the gene.

[0024] The elongations in comparison with the sequence known from the state of the art consist in SEQ ID No. 3 of the base-pairs 1 to 508 and 1684 to 2234, respectively.

[0025] In SEQ ID No. 1 the elongations in comparison with the sequence known from the state of the art consist of the base-pairs 1 to 531 and 1621 to 2160, respectively.

[0026] The amino-acid sequences of the associated gene products are represented in SEQ ID No. 4 and SEQ ID No. 2, respectively.

[0027] It has been found that processes for attenuation that are known as such can be employed particularly successfully with the aid of the elongated sequences that are made available in this way.

[0028] Such a process is the method of gene replacement. With this method, a mutation such as, for example, a deletion, an insertion or base-exchange in the gene of interest is produced in vitro. The allele that is produced is, in turn, cloned into a vector that is non-replicative in respect of C. glutamicum and said vector is subsequently converted by transformation or conjugation into the desired host of C. glutamicum. After homologous recombination by means of a first cross-over event bringing about integration and by means of a suitable second cross-over event in the target gene or in the target sequence bringing about an excision, the incorporation of the mutation or of the allele is obtained. This method was used in EP: 00110021.3 *I.B.R.*, for example, in order to switch off the secG gene of C. glutamicum.

[0029] The elongation of the sequences that are employed is not restricted to 600 base-pairs in front of the start codon and behind the stop codon. It preferably lies within the range from 300 to 700 base-pairs, but it may also amount to up to 800 base-pairs. The elongations may also contain different quantities of base-pairs.

[0030] The sequences, described in the stated passages, coding for 6-phosphofructokinase or 1-phosphofructokinase can be used in accordance with the invention. Moreover, use can be made of alleles of 6-phosphofructokinase or 1-phosphofructokinase that arise from the degenerate nature

of the genetic code or as a result of functionally neutral sense mutations.

[0031] With a view to achieving an attenuation, either the expression of the gene coding for 6-phosphofructokinase and/or of the gene coding for 1-phosphofructokinase or the catalytic properties of the gene products can be lowered or switched off. Both measures are optionally combined.

[0032] The gene expression can be reduced by suitable culturing or by genetic modification (mutation) of the signal structures of gene expression. Signal structures of gene expression are, for example, repressor genes, activator genes, operators, promoters, attenuators, ribosome binding sites, the start codon and terminators. Data relating to this can be found by a person skilled in the art in, for example, patent application WO 96/15246, in Boyd and Murphy (Journal of Bacteriology 170: 5949 (1988)) *I.B.R.*, in Voskuil and Chambliss (Nucleic Acids Research 26: 3548 (1998), in Jensen and Hammer (Biotechnology and Bioengineering 58: 191 (1998)) *I.B.R.*, in Pátek et al. (Microbiology 142: 1297 (1996)) *I.B.R.* and in known textbooks on genetics and molecular biology such as, for example, the textbook by Knippers (*Molekulare Genetik*, 6th Edition, Georg Thieme Verlag, Stuttgart, Germany, 1995) *I.B.R.* or in that by Winnacker (*Gene und Klone*, VCH Verlagsgesellschaft, Weinheim, Germany, 1990) *I.B.R.*

[0033] Mutations that lead to a change in or a lowering of the catalytic properties of enzyme proteins are known from the state of the art; by way of examples, mention may be made of the papers by Qiu and Goodman (Journal of Biological Chemistry 272: 8611-8617 (1997)) *I.B.R.*, Sugimoto et al. (Bioscience Biotechnology and Biochemistry 61: 1760-1762 (1997)) *I.B.R.* and Möckel (*Die Threonindehydratase aus Corynebacterium glutamicum: Aufhebung der allosterischen Regulation und Struktur des Enzyms*, Berichte des Forschungszentrums Jülichs, Jül-2906,

ISSN09442952, Jülich, Germany, 1994 *I.B.R.*). Synoptic accounts can be gathered from known textbooks on genetics and molecular biology such as, for example, that by Hagemann (*Allgemeine Genetik*, Gustav Fischer Verlag, Stuttgart, 1986) *I.B.R.*

[0034] Transitions, transversions, insertions and deletions enter into consideration by way of mutations. Depending on the effect of the amino-acid exchange on the enzyme activity, one speaks of missense mutations or nonsense mutations. Insertions or deletions of at least one base-pair in a gene lead to frame-shift mutations, as a consequence of which false amino acids are incorporated or the translation terminates prematurely. Deletions of several codons typically lead to a complete loss of enzyme activity. Instructions for the generation of mutations of such a type pertain to the state of the art and can be gathered from known textbooks on genetics and molecular biology such as, for example, the textbook by Knippers (*Molekulare Genetik*, 6th Edition, Georg Thieme Verlag, Stuttgart, Germany, 1995) *I.B.R.*, that by Winnacker (*Gene und Klone*, VCH Verlagsgesellschaft, Weinheim, Germany, 1990) or that by Hagemann (*Allgemeine Genetik*, Gustav Fischer Verlag, Stuttgart, 1986) *I.B.R.*

[0035] Customary methods for mutating genes of C. glutamicum are the methods of gene disruption and of gene replacement described by Schwarzer and Pühler (Bio/Technology 9, 84-87 (1991)) *I.B.R.*

[0036] In the case of the method of gene disruption, a central part of the coding region of the gene of interest is cloned into a plasmid vector that is able to replicate in a host (typically E. coli) but not in C. glutamicum. By way of vectors, pSUP301 (Simon et al., Bio/Technology 1, 784-791 (1983) *I.B.R.*), pK18mob or pK19mob (Schäfer et al., Gene 145, 69-73 (1994) *I.B.R.*), pK18mobsacB or pK19mobsacB (Jäger et al., Journal of Bacteriology 174: 5462-65 (1992)

I.B.R.), pGEM-T (Promega corporation, Madison, WI, USA), pCR2.1-TOPO (Shuman (1994), Journal of Biological Chemistry 269:32678-84 *I.B.R.*; US Patent 5,487,993 *I.B.R.*), pCR®Blunt (Invitrogen, Groningen, Netherlands; Bernard et al., Journal of Molecular Biology, 234: 534-541 (1993) *I.B.R.*) or pEM1 (Schrumpf et al, 1991, Journal of Bacteriology 173:4510-4516 *I.B.R.*), for example, enter into consideration. The plasmid vector, which contains the central part of the coding region of the gene, is subsequently converted by conjugation or transformation into the desired strain of C. glutamicum. The method of conjugation is described, for example, in Schäfer et al. (Applied and Environmental Microbiology 60, 756-759 (1994)) *I.B.R.* Methods for transformation are described, for example, in Thierbach et al. (Applied Microbiology and Biotechnology 29, 356-362 (1988)) *I.B.R.*, Dunican and Shivnan (Bio/Technology 7, 1067-1070 (1989)) *I.B.R.* and Tauch et al. (FEMS Microbiological Letters 123, 343-347 (1994)) *I.B.R.* After homologous recombination by means of a cross-over event, the coding region of the gene in question is interrupted by the vector sequence, and two incomplete alleles are obtained, from each of which the 3'-end or the 5'-end is missing. This method was used, for example, by Fitzpatrick et al. (Applied Microbiology and Biotechnology 42, 575-580 (1994)) *I.B.R.* for the purpose of switching off the recA gene of C. glutamicum.

[0037] In the case of the method of gene replacement, a mutation such as, for example, a deletion, an insertion or a base- exchange in the gene of interest is produced in vitro. The allele that is produced is, in turn, cloned into a vector that is non-replicative in respect of C. glutamicum and said vector is subsequently converted by transformation or conjugation into the desired host of C. glutamicum. After homologous recombination by means of a first cross-over event bringing about integration and by means of a suitable second cross-over event in the target

gene or in the target sequence bringing about an excision, the incorporation of the mutation or of the allele is obtained. This method was used, for example, by Peters-Wendisch et al. (Microbiology 144, 915 - 927 (1998)) *I.B.R.*, in order to switch off the pyc gene of C. glutamicum by a deletion.

[0038] In this way a deletion, an insertion or a base-exchange can be incorporated into the gene coding for 6-phosphofructokinase and/or the gene coding for 1-phosphofructokinase.

[0039] In addition, for the production of L-amino acids it can be advantageous, in addition to the attenuation of the gene coding for 6-phosphofructokinase and/or of the gene coding for 1-phosphofructokinase, to enhance, in particular to overexpress, one or more enzymes of the respective biosynthetic pathway, of glycolysis, of anaplerotic reactions, of the citric-acid cycle, of the pentose-phosphate cycle, of the export of amino acid and optionally regulatory proteins.

[0040] The term 'enhancement' or in this context describes the increase in the intracellular activity of one or more enzymes or proteins in a micro-organism which are coded by the corresponding DNA by, for example, the copy-number of the gene or genes being increased, by use being made of a strong promoter or a gene that codes for a corresponding enzyme or protein with a high activity and by optionally combining these measures.

[0041] By virtue of the measures of enhancement, in particular overexpression, the activity or concentration of the corresponding protein is increased in general by at least 10%, 25%, 50%, 75%, 100%, 150%, 200%, 300%, 400% or 500%, maximally up to 1000% or 2000%, relative to that of the wild-type protein or of the activity or concentration of the protein in the initial micro-organism.

[0042] The use of endogenous genes is generally preferred. The term "endogenous genes" or "endogenous nucleotide sequences" is to be understood to mean the genes or nucleotide sequences, respectively, existing in the population of a species.

[0043] Thus, for the preparation of L-lysine, in addition to the attenuation of the gene coding for 6-phosphofructokinase and/or of the gene coding for 1-phosphofructokinase, one or more of the genes selected from the group comprising

- the gene lysC coding for a feedback-resistant aspartate kinase (Accession No. P26512, EP-B-0387527 *I.B.R.*; EP-A-0699759 *I.B.R.*; WO 00/63388 *I.B.R.*),
- the gene dapA coding for dihydrodipicolinate synthase (EP-B 0 197 335 *I.B.R.*),
- the gene gap coding for glyceraldehyde-3-phosphate dehydrogenase (Eikmanns (1992), Journal of Bacteriology 174:6076-6086 *I.B.R.*),
- simultaneously the gene pyc coding for pyruvate carboxylase (DE-A-198 31 609 *I.B.R.*),
- the gene mqo coding for malate:quinone oxidoreductase (Molenaar et al., European Journal of Biochemistry 254, 395 - 403 (1998) *I.B.R.*),
- the gene zwf coding for glucose-6-phosphate dehydrogenase (JP-A-09224661 *I.B.R.*),
- simultaneously the gene lysE coding for the lysine-export protein (DE-A-195 48 222 *I.B.R.*),
- the gene zwa1 coding for the zwa1 protein (DE: 19959328.0 *I.B.R.*, DSM 13115),

- the gene tpi coding for triosephosphate isomerase (Eikmanns (1992), Journal of Bacteriology 174:6076-6086 *I.B.R.*), and
- the gene pgk coding for 3-phosphoglycerate kinase (Eikmanns (1992), Journal of Bacteriology 174:6076-6086 *I.B.R.*)

is/are enhanced, in particular overexpressed.

[0044] Moreover, for the production of amino acids, in particular L-lysine, it can be advantageous, in addition to the attenuation of the gene coding for 6-phosphofructokinase and/or of the gene coding for 1-phosphofructokinase, simultaneously to attenuate, in particular to reduce the expression of, one or more of the genes selected from the group comprising

- the gene pck coding for phosphoenolpyruvate carboxykinase (DE 199 50 409.1 *I.B.R.*, DSM 13047),
- the gene pgi coding for glucose-6-phosphate isomerase (US 09/396,478 *I.B.R.*, DSM 12969),
- the gene poxB coding for pyruvate oxidase (DE:1995 1975.7 *I.B.R.*, DSM 13114),
- the gene fda coding for fructose bisphosphate aldolase (Mol. Microbiol. 3 (11), 1625-1637 (1989) *I.B.R.*; gene bank Accession Number X17313) and
- the gene zwa2 coding for the zwa2 protein (DE: 19959327.2 *I.B.R.*, DSM 13113).

[0045] Finally, for the production of amino acids it can be advantageous, in addition to the attenuation of the gene coding for 6-phosphofructokinase and/or of the gene coding for 1-phosphofructokinase, to exclude undesirable side reactions (Nakayama: *Breeding of Amino Acid Producing*

Micro-organisms, in: *Overproduction of Microbial Products*, Krumphanzl, Sikyta, Vanek (eds.), Academic Press, London, UK, 1982 *I.B.R.*).

[0046] The micro-organisms that are produced in accordance with the invention are likewise a subject of the invention and can be cultivated continuously or discontinuously in the batch process (batch cultivation) or in the fed-batch or repeated-fed-batch process for the purpose of producing L-amino acids. A summary of known cultivation methods is described in the textbook by Chmiel (*Bioprozesstechnik 1. Einführung in die Bioverfahrenstechnik* (Gustav Fischer Verlag, Stuttgart, 1991) *I.B.R.*) or in the textbook by Storhas (*Bioreaktoren und periphere Einrichtungen* (Vieweg Verlag, Braunschweig/Wiesbaden, 1994) *I.B.R.*).

[0047] The culture medium to be used has to satisfy the demands of the respective strains in suitable manner. Descriptions of culture media of various micro-organisms are contained in the manual entitled *Manual of Methods for General Bacteriology* published by the American Society for Bacteriology (Washington D.C., USA, 1981) *I.B.R.*

[0048] Sugar and carbohydrates such as, for example, glucose, saccharose, lactose, fructose, maltose, molasses, starch and cellulose, oils and fats such as, for example, soybean oil, sunflower oil, peanut oil and coconut oil, fatty acids such as, for example, palmitic acid, stearic acid and linoleic acid, alcohols such as, for example, glycerin and ethanol, and organic acids such as, for example, acetic acid can be used by way of carbon source. These substances can be used individually or as a mixture.

[0049] Organic nitrogenous compounds such as peptones, yeast extract, meat extract, malt extract, maize steep liquor, soybean flour and urea, or inorganic compounds such as ammonium sulfate, ammonium chloride, ammonium phosphate, ammonium carbonate and ammonium nitrate can be used by way

of nitrogen source. The nitrogen sources can be used individually or as a mixture.

[0050] Phosphoric acid, potassium dihydrogenphosphate or dipotassium hydrogenphosphate or the corresponding sodium-containing salts can be used by way of phosphorus source. The culture medium must, moreover, contain salts of metals such as, for example, magnesium sulfate or iron sulfate, that are necessary for growth. Finally, essential growth substances such as amino acids and vitamins can be employed in addition to the aforementioned substances. Besides, suitable precursors can be added to the culture medium. The stated feed materials can be added to the culture in the form of a single charge or can be fed in during the cultivation in suitable manner.

[0051] With a view to controlling the pH of the culture, basic compounds such as sodium hydroxide, potassium hydroxide, ammonia or ammoniacal liquor or acidic compounds such as phosphoric acid or sulfuric acid are employed in suitable manner. With a view to controlling the formation of foam, anti-foaming agents such as, for example, fatty-acid polyglycol esters can be employed. With a view to maintaining the stability of plasmids, suitable substances acting selectively, such as antibiotics for example, can be added to the medium. In order to maintain aerobic conditions, oxygen or oxygenous gas mixtures, such as air for example, are introduced into the culture. The temperature of the culture is normally around 20°C to 45°C and preferably 25°C to 40°C. The culture is continued for such time until a maximum of the desired product has formed. This objective is normally attained within a period from 10 hours to 160 hours.

[0052] Methods for the determination of L-amino acids are known from the state of the art. The analysis can be undertaken as described in Spackman et al. (Analytical Chemistry, 30, (1958), 1190 *I.B.R.*) by anion-exchange

chromatography with subsequent ninhydrin derivation, or it can be undertaken by reversed-phase HPLC, as described in Lindroth et al. (Analytical Chemistry (1979) 51: 1167-1174 *I.B.R.*).

[0053] The following micro-organism was deposited in the form of pure culture on 11 January 2002 in the Deutsche Sammlung für Mikroorganismen und Zellkulturen (German Collection of Micro-Organisms and Cell Cultures, DSMZ, Braunschweig, Germany):

- Escherichia coli DH5alphamcr/pXK99EmobpfkB (= DH5αmcr/pXK99EmobpfkB) as DSM 14741.

[0054] The present invention is elucidated in more detail in the following on the basis of exemplary embodiments.

[0055] Example 1

Preparation of a genomic cosmid gene bank from Corynebacterium glutamicum ATCC 13032

[0056] Chromosomal DNA from Corynebacterium glutamicum ATCC 13032 was isolated as described in Tauch et al. (1995, Plasmid 33:168-179) *I.B.R.* and partially cleaved with the restriction enzyme Sau3AI (Amersham Pharmacia, Freiburg, Germany, product description Sau3AI, Code No. 27-0913-02). The DNA fragments were dephosphorylated with shrimp alkaline phosphatase (Roche Diagnostics GmbH, Mannheim, Germany, product description SAP, Code No. 1758250). The DNA of the cosmid vector SuperCos1 (Wahl et al. (1987) Proceedings of the National Academy of Sciences USA 84:2160-2164 *I.B.R.*), obtained from Stratagene (La Jolla, USA, product description SuperCos1 Cosmid Vektor Kit, Code No. 251301) was cleaved with the restriction enzyme XbaI (Amersham Pharmacia, Freiburg, Germany, product description XbaI, Code No. 27-0948-02) and likewise dephosphorylated with shrimp alkaline phosphatase.

[0057] Subsequently the cosmid DNA was cleaved with the restriction enzyme BamHI (Amersham Pharmacia, Freiburg, Germany, product description BamHI, Code No. 27-0868-04). The cosmid DNA that was treated in this way was mixed with the treated ATCC13032 DNA, and the charge was treated with T4-DNA-Ligase (Amersham Pharmacia, Freiburg, Germany, product description T4-DNA-Ligase, Code No. 27-0870-04). The ligation mixture was subsequently packaged in phages with the aid of the Gigapack II XL Packing Extracts (Stratagene, La Jolla, USA, product description Gigapack II XL Packing Extract, Code No. 200217).

[0058] With a view to infection of the E. coli strain NM554 (Raleigh et al. 1988, Nucleic Acid Research 16:1563-1575 *I.B.R.*), the cells were taken up in 10 mM $MgSO_4$ and mixed with an aliquot of the phage suspension. Infection and titration of the cosmid bank were carried out as described in Sambrook et al. (1989, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor) *I.B.R.*, whereby the cells were plated onto LB agar (Lennox, 1955, Virology, 1:190 *I.B.R.*) with 100 mg/l ampicillin. After incubation overnight at 37°C, recombinant single clones were selected.

[0059] Example 2

Ascertainment of the upstream and downstream elongations of the sequences of the genes pfkA and pfkB known from the state of the art

[0060] The cosmid DNA of a single colony was isolated with the Qiaprep Spin Miniprep Kit (Product No. 27106, Qiagen, Hilden, Germany) in accordance with the manufacturer's instructions and partially cleaved with the restriction enzyme Sau3AI (Amersham Pharmacia, Freiburg, Germany, product description Sau3AI, Product No. 27-0913-02). The DNA fragments were dephosphorylated with shrimp alkaline phosphatase (Roche Diagnostics GmbH, Mannheim, Germany, product description SAP, Product No. 1758250). After gel-

electrophoretic fractionation, isolation of the cosmid fragments was effected within the size-range from 1500 to 2000 bp with the QiaExII Gel Extraction Kit (Product No. 20021, Qiagen, Hilden, Germany *I.B.R.*).

[0061] The DNA of the sequencing vector pZero-1 obtained from Invitrogen (Groningen, Netherlands, product description Zero Background Cloning Kit, Product No. K2500-01 *I.B.R.*) was cleaved with the restriction enzyme BamHI (Amersham Pharmacia, Freiburg, Germany, product description BamHI, Product No. 27-0868-04 *I.B.R.*). The ligation of the cosmid fragments into the sequencing vector pZero-1 was carried out as described by Sambrook et al. (1989, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor *I.B.R.*), whereby the DNA mixture was incubated overnight with T4-Ligase (Pharmacia Biotech, Freiburg, Germany). This ligation mixture was subsequently electroporated into the E. coli strain DH5αMCR (Grant, 1990, Proceedings of the National Academy of Sciences U.S.A., 87:4645-4649 *I.B.R.*) (Tauch et al. 1994, FEMS Microbiol Letters, 123:343-7 *I.B.R.*) and plated onto LB agar (Lennox, 1955, Virology, 1:190 *I.B.R.*) with 50 mg/l Zeocin.

[0062] The plasmid preparation of the recombinant clones was undertaken with a Biorobot 9600 (Product No. 900200, Qiagen, Hilden, Germany). Sequencing was effected in accordance with the dideoxy chain-termination method of Sanger et al. (1977, Proceedings of the National Academy of Sciences U.S.A., 74:5463-5467) *I.B.R.* with modifications in accordance with Zimmermann et al. (1990, Nucleic Acids Research, 18:1067) *I.B.R.* Use was made of the "RR dRhodamin Terminator Cycle Sequencing Kit" manufactured by PE Applied Biosystems (Product No. 403044, Weiterstadt, Germany). Gel-electrophoretic fractionation and analysis of the sequencing reaction were undertaken in a "Rotiphorese NF Acrylamid/Bisacrylamid" gel (29:1) (Product No. A124.1, Roth, Karlsruhe, Germany *I.B.R.*) with an "ABI

Prism 377" sequencer manufactured by PE Applied Biosystems (Weiterstadt, Germany).

[0063] The raw sequence data obtained were subsequently processed by applying the Staden program package (1986, Nucleic Acids Research, 14:217-231 *I.B.R.*) Version 97-0. The individual sequences of the pZero1 derivatives were assembled into a coherent contig. The computer-aided coding-region analysis was produced with the program XNIP (Staden, 1986, Nucleic Acids Research, 14:217-231 *I.B.R.*). Further analyses can be carried out with the "BLAST search program" (Altschul et al., 1997, Nucleic Acids Research, 25:3389-3402 *I.B.R.*) against the non-redundant databank of the "National Center for Biotechnology Information" (NCBI, Bethesda, MD, USA) *I.B.R.*

[0064] The relative degree of substitution or mutation in the polynucleotide or amino acid sequence to produce a desired percentage of sequence identity can be established or determined by well-known methods of sequence analysis. These methods are disclosed and demonstrated in Bishop, et al. "DNA & Protein Sequence Analysis (A Practical Approach"), Oxford Univ. Press, Inc. (1997) *I.B.R.* and by Steinberg, Michael "Protein Structure Prediction" (A Practical Approach), Oxford Univ. Press, Inc. (1997) *I.B.R.*

[0065] The known nucleotide sequences of the genes pfkA and pfkB that are extended by the upstream and downstream elongations obtained in this way are represented in SEQ ID No. 1 and SEQ ID No. 3.

[0066] Example 3

Preparation of the expression vector pXK99EmobpfkB for IPTG-induced expression of the pfkB gene in C. glutamicum

[0067] 3.1 Cloning of the pfkB gene

Chromosomal DNA is isolated from the strain ATCC 13032 in accordance with the method of Eikmanns et al. (Microbiology 140: 1817-1828 (1994)) *I.B.R.* On the basis of the known sequence of the pfkB gene for C. glutamicum the following oligonucleotides for the polymerase chain reaction are selected:

pfkB for:
5`-CT TCT AGA-CCC GAC CAC AAC TTT CAG G -3` SEQ ID NO: 5

pfkBint int:
5`- AG AAG CTT-GCC AGG TGT ATC CAA GCT CTC -3` SEQ ID NO: 6

[0068] In this connection the primers are selected in such a way that the amplified fragment contains the incomplete gene, beginning with the native ribosome binding site without promoter region, as well as the anterior region of the pfkB gene. In addition, the primer pfkB for contains the sequence for the cleavage site of the restriction endonuclease XbaI, and the primer pfkB int contains the sequence for the cleavage site of the restriction endonuclease HindIII, which are marked by underlining in the nucleotide sequences represented above.

[0069] The primers that are represented are synthesized by MWG-Biotech AG (Ebersberg, Germany), and the PCR reaction is carried out in accordance with the standard PCR method of Innis et al. (PCR Protocols. A Guide to Methods and Applications, 1990, Academic Press) *I.B.R.* with Pwo polymerase produced by Roche Diagnostics GmbH (Mannheim, Germany). With the aid of the polymerase chain reaction the primers enable the amplification of a 594-bp DNA fragment that bears the incomplete pfkB gene including the native ribosome binding site.

[0070] The 594-bp pfkB fragment is cleaved with the restriction endonucleases XbaI and HindIII and is subsequently isolated from the agarose gel with the QiaExII

Gel Extraction Kit (Product No. 20021, Qiagen, Hilden, Germany *I.B.R.*).

[0071] 3.2 Construction of the expression vector pXK99Emob

The IPTG-inducible expression vector pXK99Emob is constructed in accordance with the state of the art. The vector is based on the Escherichia coli expression vector pTRC99A (Amann et al., Gene 69: 301-315 (1988) *I.B.R.*) and contains the trc promoter which is inducible by addition of the lactose derivative IPTG (isopropyl-β-D-thiogalactopyranoside), the termination regions T1 and T2, the replication origin ColE1 from E. Coli, the $lacI^q$ gene (repressor of the lac operon of E.coli), a multiple cloning site (mcs) (Norrander, J.M. et al. Gene 26, 101-106 (1983) *I.B.R.*), the kanamycin-resistance gene aph(3´)-IIa from E. coli (Beck et al. (1982), Gene 19: 327-336 *I.B.R.*) and the RP4 mobilization site from the cloning vector pK18mobsacB (Schäfer et al., Gene 14: 69-73 (1994) *I.B.R.*).

[0072] It has been found that the vector pXK99Emob is quite especially suitable for regulating the expression of a gene, in particular for bringing about the attenuated expression in coryneform bacteria. The vector pXK99Emob is an E. coli expression vector and can be employed in E. coli for the enhanced expression of a gene.

[0073] Since the vector cannot replicate independently in coryneform bacteria, it is preserved in the cell only when it integrates into the chromosome. The peculiarity of this vector in this connection is the use for the regulated expression of a gene after cloning of a gene segment from the anterior region of the corresponding gene into the vector, containing the start codon and the native ribosome binding site, and after subsequent integration of the vector in coryneform bacteria, in particular C. glutamicum. By addition of metered amounts of IPTG to the nutrient

medium the gene expression is regulated. In this connection, quantities from 0.5 µM up to 10 µM IPTG bring about a very weak expression of the corresponding gene, and quantities from 10 µM up to 100 µM bring about a slightly attenuated to normal expression of the corresponding gene.

[0074] The constructed E. coli expression vector pXK99Emob is transferred by means of electroporation (Tauch et al. 1994, FEMS Microbiol Letters, 123: 343-347 *I.B.R.*) into E. coli DH5αmcr (Grant, 1990, Proceedings of the National Academy of Sciences U.S.A., 87:4645-4649 *I.B.R.*). Selection of the transformants is undertaken on LB agar (Sambrook et al., Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1989 *I.B.R.*) that has been supplemented with 50 mg/l kanamycin.

[0075] Plasmid DNA is isolated from a transformant in accordance with the customary methods (Peters-Wendisch et al., 1998, Microbiology, 144, 915 - 927 *I.B.R.*), is cut with the restriction endonuclease NcoI, and the plasmid is examined by subsequent agarose-gel electrophoresis.

[0076] The plasmid construct that is obtained in this way is designated as pXK99Emob (Figure 1). The strain that is obtained by electroporation of the plasmid pXK99Emob into the E. coli strain DH5αmcr is called E. coli DH5αmcr/pXK99Emob.

[0077] 3.3 Cloning of the pfkB fragment into the E. coli expression vector pXK99Emob

By way of vector, use is made of the E. coli expression vector pXK99Emob described in Example 3.2. DNA of this plasmid is cleaved completely with the restriction enzymes XbaI and HindIII and is subsequently dephosphorylated with shrimp alkaline phosphatase (Roche Diagnostics GmbH, Mannheim, Germany, product description SAP, Product No. 1758250 *I.B.R.*).

[0078] The approximately 580-bp pfkB fragment described in Example 3.1, which is obtained by means of PCR and cleaved with the restriction endonucleases XbaI and HindIII, is mixed with the prepared vector pXK99Emob, and the charge is treated with T4-DNA-Ligase (Amersham Pharmacia, Freiburg, Germany, product description T4-DNA-Ligase, Code No. 27-0870-04 *I.B.R.*). The ligation charge is transformed into the E. coli strain DH5αmcr (Hanahan, In: DNA Cloning. A Practical Approach, Vol. I, IRL Press, Oxford, Washington DC, USA *I.B.R.*). Selection of plasmid-bearing cells is undertaken by plating the transformation charge onto LB agar (Lennox, 1955, Virology, 1:190 *I.B.R.*) with 50 mg/l kanamycin. After incubation overnight at 37°C, recombinant individual clones are selected. Plasmid DNA is isolated from a transformant with the Qiaprep Spin Miniprep Kit (Product No. 27106, Qiagen, Hilden, Germany *I.B.R.*) in accordance with the manufacturer's instructions and is cleaved with the restriction enzymes XbaI and HindIII, in order to examine the plasmid by subsequent agarose-gel electrophoresis. The plasmid that is obtained is called pXK99EmobpfkB. It is represented in Figure 2.

[0079] Example 4

Integration of the vector pXK99EmobpfkB into the genome of the C. glutamicum strain DSM5715

[0080] The vector pXK99EmobpfkB named in Example 3 is electroporated into the strain C. glutamicum DSM5715 in accordance with the electroporation method of Tauch et al. (1989 FEMS Microbiology Letters 123: 343-347) *I.B.R.* The vector cannot replicate independently in DSM5715 and is preserved in the cell only when it has integrated into the chromosome. Selection of clones with integrated pXK99EmobpfkB is undertaken by plating the electroporation charge onto LB agar (Sambrook et al., Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor, New York,

1989 *I.B.R.*) that has been supplemented with 15 mg/l kanamycin and IPTG (1 mM).

[0081] A selected kanamycin-resistant clone that has inserted the plasmid pXK99EmobpfkB named in Example 3 within the chromosomal pfkB gene of DSM5715 is designated as DSM5715::pXK99EmobpfkB.

[0082] Example 5

Preparation of lysine

[0083] The C. glutamicum strain DSM5715::pCXK99EmobpfkB obtained in Example 4 is cultured in a nutrient medium that is suitable for the production of lysine, and the lysine content in the supernatant of the culture is determined. Addition of IPTG results in an attenuated expression of the pfkB gene, regulated by the trc promoter.

[0084] To this end, the strain is firstly incubated for 24 hours at 33°C on agar plate with the appropriate antibiotic (brain/heart agar with kanamycin (25 mg/l) and IPTG (10 µM)). Starting from this agar-plate culture, a preculture is inoculated (10 ml medium in a 100-ml Erlenmeyer flask). The complete medium Cg III is used as medium for the preculture.

[0085]

Medium Cg III	
NaCl	2.5 g/l
Bacto-peptone	10 g/l
Bacto-yeast extract	10 g/l
Glucose (autoclaved separately)	2% (w/v)

The pH value was adjusted to pH 7.4

To this medium there are added kanamycin (25 mg/l) and IPTG (10 µM). The preculture is incubated on the shaker for 16 hours at 33°C at 240 rpm. A main culture is inoculated from this preculture, so that the initial OD (660 nm) of the main culture amounts to 0.1. The medium MM is used for the main culture.

[0086] To this medium there were added kanamycin (25 mg/l) and IPTG (10 µM). The preculture was incubated on the shaker for 16 hours at 33°C at 240 rpm. The OD (660) of the preculture amounted to 14.7. 68 µl from this preculture were inoculated into a main culture, so that the initial OD (660 nm) of the main culture amounted to 0.1. By virtue of the transfer of IPTG-containing medium from the preculture, the IPTG concentration in the main culture amounted to about 0.07 µM/l. The medium MM was used for the main culture.

[0087] Medium MM

CSL (corn steep liquor)	5 g/l
MOPS (morpholinopropanesulfonic acid)	20 g/l
Glucose (autoclaved separately)	50 g/l
Salts:	
$(NH_4)_2SO_4$	25 g/l
KH_2PO_4	0.1 g/l
$MgSO_4 * 7 H_2O$	1.0 g/l
$CaCl_2 * 2 H_2O$	10 mg/l
$FeSO_4 * 7 H_2O$	10 mg/l
$MnSO_4 * H_2O$	5.0 mg/l

Biotin (sterilized by filtration)	0.3 mg/l
Thiamin * HCl (sterilized by filtration)	0.2 mg/l
Leucine (sterilized by filtration)	0.1 g/l
$CaCO_3$	25 g/l

CSL, MOPS and the salt solution are adjusted to pH 7 with ammoniacal liquor and are autoclaved. Subsequently the sterile substrate and vitamin solutions are added, as well as the dry-autoclaved $CaCO_3$.

[0088] Culturing is effected in 10 ml volumes in a 100-ml Erlenmeyer flask with baffles. Kanamycin (25 mg/l) is added. Culturing is effected at 33°C and at 80% atmospheric moisture.

[0089] After 48 hours the OD at a measuring wavelength of 660 nm is ascertained with a Biomek 1000 (Beckmann Instruments GmbH, Munich). The quantity of lysine that is formed is determined with an amino-acid analyzer manufactured by Eppendorf-BioTronik (Hamburg, Germany) by ion-exchange chromatography and post-column derivation with detection of ninhydrin.

[0090] The result of the experiment is represented in Table 1.

Table 1

Strain	OD (660 nm)	Lysine HCl g/l
DSM5715	12.2	15.31
DSM5715::pXK99EmobpfkB	7.8	16.89

[0091] This application claims priority to German Priority Document Application No. 101 12 992.0, filed on March 17
26, 2001. The German Priority Document is hereby incorporated by reference in its entirety.